AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EXPERIMENTAL THERAPEUTICS

Encephalitozoon cuniculi Genotype III Evinces a Resistance to Albendazole Treatment in both Immunodeficient and Immunocompetent Mice

Bohumil Sak,[a] Klára Brdíčková,[b] Nikola Holubová,[a,c] Dana Květoňová,[a] Lenka Hlásková,[a] Martin Kváč[a,c]

[a]Institute of Parasitology, Biology Centre, Czech Academy of Science, České Budějovice, Czech Republic
[b]Faculty of Science, University of South Bohemia in České Budějovice, České Budějovice, Czech Republic
[c]Faculty of Agriculture, University of South Bohemia in České Budějovice, České Budějovice, Czech Republic

ABSTRACT Of four genotypes of *Encephalitozoon cuniculi*, *E. cuniculi* genotype II is considered to represent a parasite that occurs in many host species in a latent asymptomatic form, whereas *E. cuniculi* genotype III seems to be more aggressive, and infections caused by this strain can lead to the death of even immunocompetent hosts. Although albendazole has been considered suitable for treatment of *Encephalitozoon* species, its failure in control of *E. cuniculi* genotype III infection has been reported. This study determined the effect of a 100× recommended daily dose of albendazole on an *Encephalitozoon cuniculi* genotype III course of infection in immunocompetent and immunodeficient mice and compared the results with those from experiments performed with a lower dose of albendazole and *E. cuniculi* genotype II. The administration of the regular dose of abendazole during the acute phase of infection reduced the number of affected organs in all strains of mice and absolute counts of spores in screened organs. However, the effect on genotype III was minor. Surprisingly, no substantial effect was recorded after the use of a 100× dose of albendazole, with larger reductions seen only in the number of affected organs and absolute counts of spores in all strains of mice, implying variations in albendazole resistance between these *Encephalitozoon cuniculi* genotypes. These results imply that differences in the course of infection and the response to treatment depend not only on the immunological status of the host but also on the genotype causing the infection. Understanding how microsporidia survive in hosts despite targeted antimicrosporidial treatment could significantly contribute to research related to human health.

KEYWORDS *Encephalitozoon cuniculi*, albendazole, genotype III, microsporidiosis, tolerance, treatment

Microsporidia, ubiquitous unicellular spore-forming microorganisms classified in the separate phylum Microsporidia, infect a wide range of vertebrate and invertebrate hosts, including humans (1). *Encephalitozoon cuniculi*, the first microsporidian identified in mammals and the first successfully isolated for long-term culture, is the best-studied microsporidian species (2–4). Natural infection with this microsporidian has been reported in variety of host species, and *E. cuniculi* induces a wide spectrum of pathogenesis, including encephalitis, keratoconjunctivitis, nephritis, and hepatitis in immunocompromised patients (5–7).

Based on the number of 5′-GTTT-3′ repeats in the ribosomal internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) gene, four *E. cuniculi* genotypes have been identified so far: genotype I ("rabbit"), genotype II ("mouse"), genotype III ("dog"), and genotype IV ("human") (8, 9). While *E. cuniculi* genotype II has been found in many

Citation Sak B, Brdíčková K, Holubová N, Květoňová D, Hlásková L, Kváč M. 2020. *Encephalitozoon cuniculi* genotype III evinces a resistance to albendazole treatment in both immunodeficient and immunocompetent mice. Antimicrob Agents Chemother 64:e00058-20. https://doi.org/10.1128/AAC.00058-20.

Address correspondence to Bohumil Sak, casio@paru.cas.cz.

Received 9 January 2020
Returned for modification 25 January 2020
Accepted 4 March 2020
Accepted manuscript posted online 9 March 2020
Published 21 April 2020

different species of hosts, the other genotypes has been reported less frequently. To date, the course of infection was experimentally described in detail for genotype II and genotype III (10–13), implying various levels of tolerance of murine hosts to different infection intensities caused by these two genotypes. While immunocompetent BALB/c and C57BL/6 and immunodeficient $CD4^{-/-}$ and $CD8^{-/-}$ mice have been shown to survive *E. cuniculi* genotype III infection despite an enormous and rapid spreading of infection and spore burden in their tissues without any obvious clinical signs of infection, immunodeficient severe combined immunodeficiency (SCID) mice and $CD8^{-/-}$ mice succumbed to *E. cuniculi* genotype II infection, which, however, did not show similar extents and intensities. Moreover, the results reported previously by Kotková et al. in 2017 (11) and by Sak et al. in 2020 (13) indicated elevated resistance of *E. cuniculi* genotype III to albendazole treatment, as only a slight and temporary reduction in infection extension was observed not only in immunodeficient but also in immunocompetent mice.

Benzimidazoles, including albendazole, are widely used in anthelminthic drugs in veterinary and human medicine and have been used as antifungal agents in agriculture. Albendazole is one of the most commonly used drugs for treating microsporidiosis, especially encephalitozoonosis in human (14). However, benzimidazole resistance has been suggested in some microsporidia, including *Enterocytozoon bieneusi* and *Vittaforma corneae* (15, 16).

In this study, we evaluated the effect of a 100×-elevated recommended dose of albendazole on infection caused by *E. cuniculi* genotype III in mice by the use of nested PCR and the quantitative PCR (qPCR) method, compared the results with those obtained with *E. cuniculi* genotype II and genotype III treated with a recommended dosage, and assessed the potential difference between these two *Encephalitozoon cuniculi* genotypes in albendazole resistance.

RESULTS

The acute phase of *E. cuniculi* genotype III infection in immunocompetent BALB/c mice was characterized by an increase in the number of affected organs, reaching a maximum 7 days postinfection (dpi), with an average spore burden of 1.7×10^8 spores/g of positive tissue and the maximum of 1.2×10^8 spores/g of tissue reported 14 dpi. The treatment with 20 mg albendazole led to a progressive reduction in the number of affected organs, reaching the minimum 1 week after treatment termination; thereafter, the *E. cuniculi* genotype III infection was detected only sporadically, mainly in brain (Fig. 1A), with 2.1×10^4 and 1.8×10^4 spores/g of tissue reported 77 and 105 dpi, respectively. The application of albendazole cleared the infection from all parts of intestines; also, *Encephalitozoon cuniculi* genotype III DNA was detected in fecal samples sporadically from 7 to 54 dpi, but shedding was intermittent over the course of infection (Fig. 1A), with a declining spore burden from the original 2.2×10^2 spores/g of feces to $5.1 \times 10°$ reported on the last day of shedding.

The spreading intensity of *E. cuniculi* genotype III in SCID mice was comparable to that observed in immunocompetent BALB/c mice, but the spore burden was 10-fold higher, reaching an average of 2.9×10^9 spores/g of positive tissue and a maximum of 6.4×10^9 spores/g of tissue in jejunum on 14 dpi (Fig. 1B). The treatment of infected SCID mice with 20 mg of albendazole from 14 to 28 dpi led to disappearance of microsporidia from several organs after treatment introduction and a decrease of spore burden to 3.5×10^5 and 2.8×10^3 spores/g of positive tissue reported 42 and 77 dpi, respectively. However, the parasite redisseminated starting from week 7 after treatment termination and SCID mice succumbed to the infection 2 weeks later (mean survival time [MST] = 94 ± 1.1 days), resulting in 1.4×10^9 spores/g of positive tissue on 91 dpi. While parasite DNA was detected in fecal samples every day from 5 dpi with an average concentration of 2.4×10^6 spores/g of feces, intermittent, less frequent shedding of spores in feces was recorded between 16 and 49 dpi, reaching 4.3×10^3 spores/g of feces. Thereafter, the spores were again detected in feces every day (except 63 dpi) up

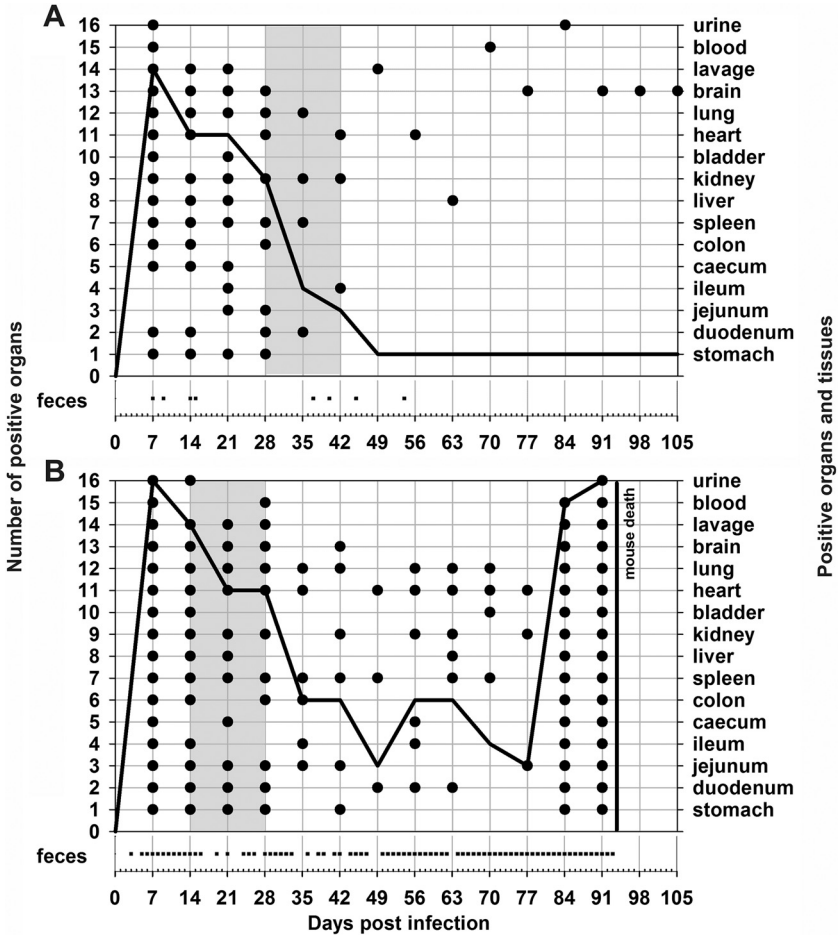

FIG 1 Course of *E. cuniculi* genotype III infection, including the pattern of spore shedding and dissemination of the infection to selected organs and tissues. (A) BALB/c mice treated with albendazole. (B) SCID mice treated with albendazole. Gray field, albendazole treatment; black line, course of *E. cuniculi* infection; dot, *E. cuniculi*-positive organ; black square, spore shedding.

to the death of the mice and the concentration rose again to up to 9.7×10^5 spores/g of feces reported at 93 dpi (Fig. 1B).

Application of 20 mg of albendazole in immunocompetent C57BL/6 mice led to a reduction in the number of affected organs starting from the first week of medication (Fig. 2A) and in a gradual decrease of spore burden from original 4.8×10^7 spores/g of positive tissue reported on 28 dpi to 3.6×10^5, 6.9×10^3, and 2.9×10^4/g of positive tissue at 42, 56, and 77 dpi, respectively. However, 4 weeks later, the microsporidia reactivated again, reaching 1.2×10^5 spores/g of positive tissue 105 dpi, and the number of affected organs grew slightly at the end of the experimental period. The treatment led to disappearance of microsporidia from most parts of intestines; also, the frequency of microsporidial DNA detection and concentration of spores in feces declined from 1.1×10^2 spores/g of feces at 21 dpi to a negative result from 43 dpi (Fig. 2A).

A comparable onset of infection was recorded for $CD4^{-/-}$ mice, reaching 2.2×10^8 spores/g of positive tissue reported on 28 dpi. However, treatment with 20 mg of albendazole had almost no effect on infection spreading and microsporidia were detected repeatedly in the majority of the screened organs (Fig. 2B). On the other hand, the medication temporarily reduced the spore burden in the organism, reaching the minimum of 4.8×10^3 spores/g of positive tissue at 56 dpi, with increases to 1.1×10^5 and 1.7×10^6 spores/g of positive tissue reported at 77 and 105 dpi, respectively. Also the shedding of microsporidia DNA in feces was not hampered by medication, only a

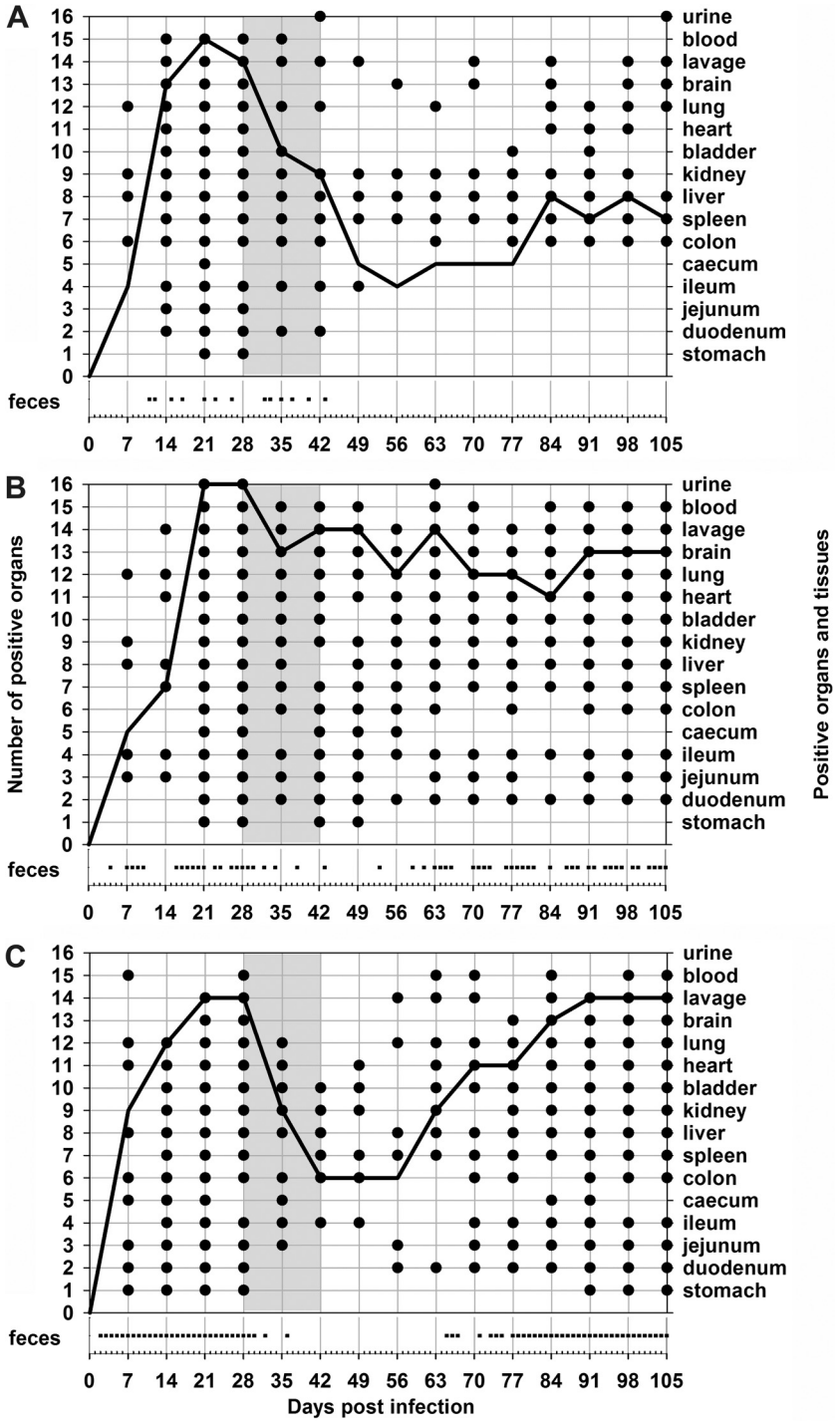

FIG 2 Course of *E. cuniculi* genotype III infection, including the pattern of spore shedding and dissemination of the infection to selected organs and tissues. (A) C57BL/6 mice treated with albendazole. (B) $CD4^{-/-}$ mice treated with albendazole. (C) $CD8^{-/-}$ mice treated with albendazole. Gray field, albendazole treatment; black line, course of *E. cuniculi* infection; dot, *E. cuniculi*-positive organ; black square, spore shedding.

slight reduction in shedding frequency and spore concentrations was monitored for 3 weeks following treatment, with the number of spores decreasing to 3.8×10^3/g of feces (Fig. 2B); however, after this period, the concentrations of spores rose again to volumes comparable to that reported before treatment (6.3×10^3/g of feces at 21 dpi versus 5.1×10^4/g of feces at 105 dpi).

Encephalitozoon cuniculi genotype III disseminated slightly more aggressively in CD8$^{-/-}$ mice than in mice lacking CD4 lymphocytes (Fig. 2C), with a maximum spore burden of 7.0×10^8/g of tissue at 28 dpi. Also, the medication led to more dynamic decrease in the number of affected organs, mainly 2 weeks after treatment termination, with the minimum spore burden of 3.3×10^4 spores/g of positive tissue reported at 56 dpi. In the weeks that followed, progressive dissemination of microsporidia was seen in most of mouse body, reaching of 8.6×10^7 spores/g of positive tissue at 105 dpi. Similarly to the results seen with CD4$^{-/-}$ mice, fecal samples were negative for microsporidia 4 weeks after treatment; however, microsporidia were thereafter detected in feces until the end of experiment at 105 dpi, nearly reaching the original concentration reported before treatment (5.9×10^4/g of feces) (Fig. 2C).

DISCUSSION

Albendazole, a benzimidazole derivate inhibiting microtubule assembly, has a broad spectrum of antihelminthic and antifungal activities. The drug binds to the colchicine binding site of β-tubulin and disrupted mitosis in sensitive organisms due to prevention of polymerization of both of the tubulin subunits, thus preventing elongation of the microtubules and depolymerization of the two subunits α-tubulin and β-tubulin (17, 18). Albendazole appeared to be effective in the treatment of microsporidiosis (19–21) and remains the preferred drug in human encephalitozoonosis (22). Albendazole was considered highly active against all of the Encephalitozoonidae (*E. hellem*, *E. cuniculi*, and *E. intestinalis*) based on *in vitro* experiments at concentrations lower than 0.1 mg/ml (23). It was also shown previously to be active in animal models of microsporidiosis and has been demonstrated to be effective in clinical cases (23). However, its effect in controlling encephalitozoonosis has been questioned based on introduction of modern diagnostic methods used in combination with experimental infections, indicating reactivation of the infection after treatment termination and spreading within host body to an extent similar to that seen before treatment (10, 11, 24).

Recently, a disproportion in the albendazole sensitivities of two *Encephalitozoon* genotypes, namely, genotype II and III, has been observed (10–13). While *E. cuniculi* genotype II infection was fully cured in immunocompetent BALB/c and C57BL/6 mice and immunodeficient CD4$^{-/-}$ mice treated with 0.2 mg albendazole (10, 12), only poor sensitivity of *E. cuniculi* genotype III to medication has been observed, resulting in milder or negligible effects of treatment in immunocompetent or immunodeficient mice, respectively (11, 13). Moreover, almost no difference has been observed between the effects of the recommended dose (0.2 mg albendazole; 12) and a 100-fold-increased dose (20 mg; present study) on infection spreading of *E. cuniculi* genotype III in C57BL/6 mice and derived knockouts (CD4$^{-/-}$, CD8$^{-/-}$); the treatment in both dosages resulted in a 10$\times$ reduction in the total spore burden, but the effect was only temporary, and the numbers of spores measured in organs several weeks posttreatment were comparable to those observed before treatment. On the other hand, treatment with increased concentration (20 mg of albendazole) of BALB/c mice infected with *E. cuniculi* genotype III resulted in elimination of infection from most of screened organs that was similar to that observed in *E. cuniculi* genotype II-infected and 0.2 mg albendazole treated-BALB/c mice (10), whereas only a limited and temporary effect was observed after medication with 0.2 mg of albendazole (11). The 100-fold-increased dose used in the present study did not prevent the SCID mice from death; only a significant prolongation of mean survival time compared to that seen with the recommended dosage (79 dpi versus 94 dpi) has been reported (11). The interstrain variation in sensitivity of mice to *E. cuniculi* genotype III infection and treatment was attributed to cellular intrinsic factors of target cells on the one hand and immunological responses on the other. These strains were shown to differ in their patterns of immune responses and in their stress sensitivities (25–27). C57BL/6 mice are generally considered T helper 1 (Th1)-biased mice, resulting in a predominance of cell-mediated immune response, while BALB/c mice are TH-2 biased, with an efficient humoral response dominance (28).

Moreover, it is already known that C57BL/6 and BALB/c mice differ in their microbiota content (29, 30).

It is well documented that frequent and prolonged use of benzimidazole drugs such as albendazole and mebendazole could lead to the emergence of anthelmintic resistance such as has occurred in nematodes of livestock (31). An association of drug resistance with single nucleotide polymorphisms (SNP) in the β-tubulin isotype 1 gene was determined previously (32).

Encephalitozoon genotype I β-tubulin has the amino acid sequence associated with sensitivity to benzimidazoles (33). However, *Enterocytozoon* (15) and *Vittaforma* (16) β-tubulins have been shown to demonstrate the presence of amino acids associated with albendazole resistance. *E. bieneusi* β-tubulin has only five of the six amino acids that were reported previously to be associated with benzimidazole activity (His_6, Phe_{167}, Glu_{198}, Phe_{200}, and Arg_{241}; numbering based on the *S. cerevisiae* sequence) (34, 35). Moreover, glutamine at position 198 is substituted for glutamic acid (15). Generally, organisms resistant to benzimidazole, such as *Entamoeba histolytica*, *Cryptosporidium parvum* and *Acanthamoeba polyphaga*, lack either or both of Glu_{198} or $Phe_{200.}$ Also, helminths, fungi, and humans have changes at Phe_{200} (36, 37). The resistance of *E. cuniculi* genotype III reported from the present study could be caused by such nucleotide polymorphism in β-tubulin sequence, but the phenomenon needs to be studied in detail in the future. Moreover, Davidse and Flach (38) and Jung at al. (35) suggested previously that the resistance conferred by a His_6-to-Leu mutation in another fungal species, *Aspergillus nidulans*, results from a direct effect on benzimidazole binding and not from, for example, an indirect increase in microtubule stability.

The results of the present study confirmed the hypothesis concerning variations in pathology and albendazole resistance of different genotypes of *Encephalitozoon* that had previously been suggested (11, 12, 39, 40). Although there was a disappearance of *E. cuniculi* genotype III from many organs during the next several weeks after 20 mg albendazole treatment, the number of positive-testing organs seen in the chronic stage of infection was much higher than that seen under conditions of chronic infection caused by *E. cuniculi* genotype II after treatment with 0.2 mg, mainly in C57BL/6 mice and derived knockouts (10, 12). Moreover, the amount of microsporidial spores in the organs was about 1 order higher than the amount seen with *E. cuniculi* genotype II.

Another surprising discovery was the finding of dose-dependent intergenotype variations in the rates of disappearance of the parasite from the gastrointestinal tract of immunocompetent BALB/c and C57BL/6 mice after treatment. While microsporidia vanished from the gastrointestinal tract in BALB/c mice following the recommended treatment (0.2 mg albendazole; 11), the infection almost cleared from gastrointestinal tract in C57BL/6 mice in response to the 100-fold-increased dose, but not the recommended dose (12). This effect could be caused by albendazole itself directly in the gut, as albendazole is poorly absorbed from the gastrointestinal tract due to its low aqueous solubility, its concentrations are negligible or undetectable in plasma, and it is rapidly converted to the sulfoxide metabolites, mainly albendazole sulfoxide, prior to reaching the systemic circulation (41). The rates of absorption of albendazole after oral administration differ among species, with 1% to 5% of the drug being successfully absorbed in humans, 20% to 30% in mice, and 50% in cattle. Oral bioavailability appears to be enhanced when albendazole is coadministered with a fatty meal, as evidenced by data showing up to 5-fold-increased plasma concentrations of albendazole sulfoxide compared to the fasted state (42). However, our data showed a simultaneous microsporidial spore number increase in organs outside the gastrointestinal tract, which is in accordance with results reported from previous studies, where the application of antimicrosporidial drugs or of potentially effective selected plant extracts led to a considerable shift of infection toward organs outside the gastrointestinal tract (10, 11, 24, 43), causing misleading disappearances of microsporidia from the gastrointestinal tract and reductions in spore shedding despite their survival in multiple organs. Similarly, immune reconstitution with antiretroviral therapies in humans greatly reduced the occurrence of microsporidiosis-associated clinical symptoms in persons with HIV infection

TABLE 1 Design of experiments[c]

Group	n1	n2	No. of sacrificed mice on dpi: 0	7	14	21	28	35	42	49	56	63	70	77	84	91	98	105
BALB/c																		
Uninfected control[a]	48	48	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Infection[b] + treatment (28–42 dpi)	48	48	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
SCID																		
Uninfected control[a]	48	48	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Infection[b] + treatment (14–28 dpi)	48	42	3	3	3	3	3	3	3	3	3	3	3	3	3	3	×	×
C57Bl/6																		
Uninfected control[a]	48	48	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Infection[b] + treatment (28–42 dpi)	48	48	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
$CD4^{-/-}$																		
Uninfected control[a]	48	48	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Infection[b] + treatment (28–42 dpi)	48	48	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
$CD8^{-/-}$																		
Uninfected control[a]	48	48	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Infection[b] + treatment (28–42 dpi)	48	48	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

[a]Inoculation with 0.2 ml sterilized deionized water.
[b]Infection with 107 *E. cuniculi* spores in 0.2 ml of sterilized deionized water.
[c]n1, number of animals used; n2, number of animals dissected; ×, not observed due to the death of the animal; dpi, day postinfection. Data highlighted in gray indicate albendazole treatment (20.0 mg/animal/day).

(44, 45), but, for obvious reasons, we can only speculate about the suggested possibility of drifting of microsporidia between the gastrointestinal tract and visceral organs.

This study was designed to deepen our knowledge about treatment of latent microsporidiosis and differences of *Encephalitozoon* genotypes. This information will help to increase understanding of the course of infection of these pathogens. The molecular background of resistance to albendazole treatment of *E. cuniculi* genotype III should be the subject of further studies.

MATERIALS AND METHODS

Ethics statement. All experimental procedures complied with the relevant law of the Czech Republic (CR; act no. 246/1992 Coll., on the protection of animals against cruelty). The study design was approved by ethical committees at the Biology Centre of CAS, the State Veterinary Administration, and the Central Commission for Animal Welfare under protocol no. 100/2016.

Mice. Immunodeficient SCID mice (strain C.B-17) of the BALB/c background, $CD4^{-/-}$ and $CD8^{-/-}$ mice of the C57BL/6 background, and immunocompetent BALB/c and C57BL/6 mice were originally obtained from Charles River, Sulzfeld, Germany, and bred in plastic cages with sterilized wood-chip bedding situated in IVC Air Handling Solutions (Techniplast, Italy) with high-efficiency particulate air (HEPA) filters. The experimental 8-week-old animals were housed in plastic cages with sterilized wood-chip bedding situated in flexible film isolators (BEM Znojmo, Czech Republic) with HEPA filters. All mice were supplied a sterilized diet (Top-Velaz, Prague, Czech Republic) and sterilized water *ad libitum*.

Parasite. The spores of *Encephalitozoon cuniculi* genotype III were originally isolated from pet rodent Steppe lemmings (*Lagurus lagurus*) (46) and were grown *in vitro* in green monkey kidney cells (Vero; line E6) maintained in RPMI 1640 medium (Sigma) supplemented with 2.5% heat-inactivated fetal bovine serum. Spores were harvested and purified from cells by centrifugation over 50% Percoll (Sigma) at 1,100 × *g* for 30 min and washed three times in deionized water before being stored in deionized water supplemented with antibiotics (Sigma; 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B) at 4°C. The spores were washed with deionized water before use.

Drugs. Aldifal (Mevak Nitra, SR), containing 100 g of albendazole in 1,000 ml, was p.o. (*per os* [orally]) administered by intragastric gavage per mouse in a dose consisting of 200 μl of pure albendazole (20 mg).

Experimental protocol. Groups of immunocompetent BALB/c and C57BL/6 mice and of immunodeficient SCID, $CD4^{-/-}$, and $CD8^{-/-}$ mice were p.o. infected with a dose of 10^7 *E. cuniculi* strain III spores in 0.2 ml of deionized water by intragastric gavage and treated daily with 20 mg of albendazole within a period of 14 days starting 14 dpi in SCID mice and 28 dpi in the rest of the mice. The course of infection and effect of treatment were compared with those seen with untreated and 0.2 mg albendazole-treated animals (11, 13). Noninfected mice of all strains were used as negative controls (Table 1). The fecal samples were obtained daily from each mouse separately and stored at −20°C for further molecular analyses. The health, mortality, and morbidity of the animals were recorded twice a day. Since the mean

survival time (MST) of individual experimental groups of animals needed to be determined, the animals were allowed to die without euthanasia to minimalize distortions resulting from variability of survival time of mice in the last phase of the infection. Three mice from each group were euthanized by cervical dislocation every seventh day postinfection, and sterile samples were obtained using the following sources and methods: urine by bladder catheterization, blood from retro-orbital sinus, peritoneal lavage with cold sterile phosphate-buffered saline (PBS), and sampling from various organs (stomach, duodenum, ileum, jejunum, cecum, colon, liver, spleen, kidney, bladder, lung, heart, and brain). Each organ was removed using a different pair of sterile dissection tools.

DNA isolation. Fecal, organ, and body fluid samples were homogenized by bead disruption using a FastPrep–24 instrument (MP Biomedicals, CA, USA) and 0.5-mm-diameter glass beads (Biospec Products, Inc., Bartlesville, OK, USA) at the speed of 5.5 m/s for 1 min. Total DNA was extracted using a commercial column-based isolation kit (GeneAll Exgene stool DNA minikit; GeneAll Biotechnology, Co., Ltd., Seoul, South Korea) or a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) as appropriate. The acquired DNA was stored at −20°C.

PCR amplification. Nested PCR protocols were used for amplification of partial sequence of 16S rRNA using microsporidian-specific primers as described by De Bosschere et al. in 2007 (47) and Katzwinkel-Wladarsch et al. in 1996 (48). The upstream primers M2F (CGG AGA GGA AGC CTT AGA GA) and MFNest (GAG AGA TGG CTA CTA CGT CCA AGG) were targeted to the 3′ region of the small-subunit (SSU) coding segment of *E. cuniculi*. The downstream primers M2R (ATA GTG ACG GGC GGT GTG T) and MSP1R (ACA GGG ACM CAT TCA) were targeted to the 5′ region of the coding segment of *E. cuniculi*. DNA obtained from spores of *E. intestinalis* grown *in vitro* in Vero E6 was used as a positive control. Ultrapure water was used as a negative control. For both PCR steps, a total of 35 cycles, each consisting of 94°C for 45 s, 58°C for 45 s, and 72°C for 60 s, were performed. Initial incubation at 94°C for 3 min, final extension at 72°C for 7 min, and a final soak at 4°C were included. PCR products were visualized on a 1% agarose gel containing 0.2 μg/ml ethidium bromide. A total of 3 randomly selected positive samples from each animal were sequenced to verify the identity with the inoculum.

Real-time quantitative PCR (qRT-PCR). Selected DNA samples were processed using a quantitative RT-PCR protocol, amplifying a 268-bp region of the 16S rRNA gene of *E. cuniculi* (49) and a 137-bp region of the murine β-actin as a housekeeping gene (50). Primers and hybridization probe labeled with fluorescein reporter and FastStart Universal Probe Master were purchased from Generi Biotech (Hradec Králové, CR) and Roche (Praha, CR). Each run included negative controls consisting of unspiked specimens and diluent blanks. Positive results were determined based on mathematical algorithms included with a LightCycler system. Results were deemed positive when the fluorescence signal crossed at the baseline at $\leq$43 cycles. The total amount of spores in 1 g of individual fecal samples or tissue samples was calculated based on a standard curve derived from serial dilutions of spores in water and feces and the known weight of fecal samples or were recalculated based on the number of β-actin copies in the tissue sample, respectively.

Statistical analysis. The statistically significant differences in the survival times of different groups of mice and the differences between the numbers of affected organs and the spore burden in individual groups of mice were analyzed by the nonparametric Mann-Whitney U test using Statistica 6.0 software (StatSoft CR, Prague, Czech Republic).

ACKNOWLEDGMENT

The study was supported by a grant from the Grant Agency of the Czech Republic (17-12871S).

REFERENCES

1. Weber R, Bryan RT, Schwartz DA, Owen RL. 1994. Human microsporidial infections. Clin Microbiol Rev 7:426–461. https://doi.org/10.1128/cmr.7.4.426.
2. Wright JH, Craighead EM. 1922. Infectious motor paralysis in young rabbits. J Exp Med 36:135–140. https://doi.org/10.1084/jem.36.1.135.
3. Levaditi C, Nicolau S, Schoen R. 1923. L'agent étiologique de l'encéphalite épizootique du lapin (*Encephalitozoon cuniculi*). C R Soc Biol Paris 89:984–986.
4. Shadduck JA. 1969. *Nosema cuniculi*: *in vitro* isolation. Science 166:516–517. https://doi.org/10.1126/science.166.3904.516.
5. Hocevar SN, Paddock CD, Spak CW, Rosenblatt R, Diaz-Luna H, Castillo I, Luna S, Friedman GC, Antony S, Stoddard RA, Tiller RV, Peterson T, Blau DM, Sriram RR, da Silva A, de Almeida M, Benedict T, Goldsmith CS, Zaki SR, Visvesvara GS, Kuehnert MJ, Microsporidia Transplant Transmission Investigation Team. 2014. Microsporidiosis acquired through solid organ transplantation: a public health investigation. Ann Intern Med 160:213–220. https://doi.org/10.7326/M13-2226.
6. Abu-Akkada SS, El Kerdany ED, Mady RF, Diab RG, Khedr GA, Ashmawy KI, Lotfy WM. 2015. *Encephalitozoon cuniculi* infection among immunocompromised and immunocompetent humans in Egypt. Iran J Parasitol 10:561–570.
7. Kicia M, Wesolowska M, Kopacz Z, Jakuszko K, Sak B, Květonová D, Krajewska M, Kváč M. 2016. Prevalence and molecular characteristics of urinary and intestinal microsporidia infections in renal transplant recipients. Clin Microbiol Infect 22:462.e5–e9. https://doi.org/10.1016/j.cmi.2016.01.014.
8. Didier ES, Vossbrinck CR, Baker MD, Rogers LB, Bertucci DC, Shadduck JA. 1995. Identification and characterization of three *Encephalitozoon cuniculi* genotypes. Parasitology 111:411–421. https://doi.org/10.1017/S0031182000065914.
9. Talabani H, Sarfati C, Pillebout E, van Gool T, Derouin F, Menotti J. 2010. Disseminated infection with a new genovar of *Encephalitozoon cuniculi* in a renal transplant recipient. J Clin Microbiol 48:2651–2653. https://doi.org/10.1128/JCM.02539-09.
10. Kotková M, Sak B, Květoňová D, Kváč M. 2013. Latent microsporidiosis caused by *Encephalitozoon cuniculi* in immunocompetent hosts: a murine model demonstrating the ineffectiveness of the immune system and treatment with albendazole. PLoS One 8:e60941. https://doi.org/10.1371/journal.pone.0060941.
11. Kotková M, Sak B, Hlásková L, Kváč M. 2017. The course of infection caused by *Encephalitozoon cuniculi* genotype III in immunocompetent and immunodeficient mice. Exp Parasitol 182:16–21. https://doi.org/10.1016/j.exppara.2017.09.022.
12. Sak B, Kotková M, Hlásková L, Kváč M. 2017. Limited effect of adaptive immune response to control encephalitozoonosis. Parasite Immunol 39:e12496. https://doi.org/10.1111/pim.12496.

13. Sak B, Brdíčková K, Holubová N, Květoňová D, Hlásková L, Kváč M. A massive systematic infection of Encephalitozoon cuniculi genotype III in mice does not cause clinical signs. PLoS One, in press.
14. Didier ES, Maddry JA, Brindley PJ, Stovall ME, Didier PJ. 2005. Therapeutic strategies for human microsporidia infections. Expert Rev Anti Infect Ther 3:419–434. https://doi.org/10.1586/14787210.3.3.419.
15. Akiyoshi DE, Weiss LM, Feng X, Williams BA, Keeling PJ, Zhang Q, Tzipori S. 2007. Analysis of the beta-tubulin genes from *Enterocytozoon bieneusi* isolates from a human and rhesus macaque. J Eukaryot Microbiol 54: 38–41. https://doi.org/10.1111/j.1550-7408.2006.00140.x.
16. Franzen C, Salzberger B. 2008. Analysis of the beta-tubulin gene from *Vittaforma corneae* suggests benzimidazole resistance. Antimicrob Agents Chemother 52:790–793. https://doi.org/10.1128/AAC.00928-07.
17. Cruz MC, Edlind T. 1997. β-Tubulin genes and the basis for benzimidazole sensitivity of the opportunistic fungus *Cryptococcus neoformans*. Microbiology 143:2003–2008. https://doi.org/10.1099/00221287-143-6-2003.
18. Costa SF, Weiss LM. 2000. Drug treatment of microsporidiosis. Drug Resist Updat 3:384–399. https://doi.org/10.1054/drup.2000.0174.
19. Gritz DC, Holsclaw DS, Neger RE, Whitcher JP, Jr, Margolis TP. 1997. Ocular and sinus microsporidial infection cured with systemic albendazole. Am J Ophthalmol 124:241–243. https://doi.org/10.1016/s0002-9394(14)70792-5.
20. Katiyar SK, Edlind TD. 1997. *In vitro* susceptibilities of the AIDS-associated microsporidian *Encephalitozoon intestinalis* to albendazole, its sulfoxide metabolite, and 12 additional benzimidazole derivatives. Antimicrob Agents Chemother 41:2729–2732. https://doi.org/10.1128/AAC.41.12.2729.
21. Johny S, Whitman DW; Bridge Study Group. 2008. Effect of four antimicrobials against an *Encephalitozoon* sp. (Microsporidia) in a grasshopper host. Parasitol Int 57:362–367. https://doi.org/10.1016/j.parint.2008.03.002.
22. Weber R, Deplazes P, Flepp M, Mathis A, Baumann R, Sauer B, Kuster H, Lüthy R. 1997. Cerebral microsporidiosis due to *Encephalitozoon cuniculi* in a patient with human immunodeficiency virus infection. N Engl J Med 336:474–478. https://doi.org/10.1056/NEJM199702133360704.
23. Didier ES. 1997. Effects of albendazole, fumagillin, and TNP-470 on microsporidial replication *in vitro*. Antimicrob Agents Chemother 41: 1541–1546. https://doi.org/10.1128/AAC.41.7.1541.
24. Lallo MA, da Costa LF, de Castro JM. 2013. Effect of three drugs against *Encephalitozoon cuniculi* infection in immunosuppressed mice. Antimicrob Agents Chemother 57:3067–3071. https://doi.org/10.1128/AAC.00157-13.
25. Engler H, Zawatzky R, Kirchner H, Armerding D. 1982. Experimental infection of inbred mice with herpes simplex virus. IV. Comparison of interferon production and natural killer cell activity in susceptible and resistant adult mice. Arch Virol 74:239–247. https://doi.org/10.1007/bf01314157.
26. Beil WJ, Meinardus-Hager G, Neugebauer DC, Sorg C. 1992. Differences in the onset of the inflammatory response to cutaneous leishmaniasis in resistant and susceptible mice. J Leukoc Biol 52:135–142. https://doi.org/10.1002/jlb.52.2.135.
27. Chiodini RJ1, Buergelt CD. 1993. Susceptibility of BALB/c, C57/B6 and C57/B10 mice to infection with *Mycobacterium paratuberculosis*. J Comp Pathol 109:309–319. https://doi.org/10.1016/S0021-9975(08)80295-2.
28. Karp JD, Cohen N, Moynihan JA. 1994. Quantitative differences in interleukin-2 and interleukin-4 production by antigen-stimulated splenocytes from individually- and group-housed mice. Life Sci 55: 789–795. https://doi.org/10.1016/0024-3205(94)00562-1.
29. Fransen F, Zagato E, Mazzini E, Fosso B, Manzari C, El Aidy S, Chiavelli A, D'Erchia AM, Sethi MK, Pabst O, Marzano M, Moretti S, Romani L, Penna G, Pesole G, Rescigno M. 2015. BALB/c and C57BL/6 mice differ in polyreactive IgA abundance, which impacts the generation of antigen-specific IgA and microbiota diversity. Immunity 43:527–540. https://doi.org/10.1016/j.immuni.2015.08.011.
30. Damlund DS, Metzdorff SB, Hasselby JP, Wiese M, Lundsager M, Nielsen DS, Buschard KS, Hansen AK, Frøkiær H. 2016. Postnatal hematopoiesis and gut microbiota in NOD mice deviate from C57BL/6 mice. J Diabetes Res 2016:6321980. https://doi.org/10.1155/2016/6321980.
31. Vlaminck J, Cools P, Albonico M, Ame S, Ayana M, Bethony J, Cringoli G, Dana D, Keiser J, Maurelli MP, Montresor A, Mekonnen Z, Mirams G, Corrêa-Oliveira R, Prichard R, Rashwan N, Rinaldi L, Sayasone S, Thomas E, Verweij JJ, Vercruysse J, Levecke B. 2018. Comprehensive evaluation of stool-based diagnostic methods and benzimidazole resistance markers to assess drug efficacy and detect the emergence of anthelmintic resistance: a Starworms study protocol. PLoS Negl Trop Dis 12:e0006912. https://doi.org/10.1371/journal.pntd.0006912.
32. Rashwan N, Bourguinat C, Keller K, Gunawardena NK, de Silva N, Prichard R. 2016. Isothermal diagnostic assays for monitoring single nucleotide polymorphisms in *Necator americanus* associated with benzimidazole drug resistance. PLoS Negl Trop Dis 10:e0005113. https://doi.org/10.1371/journal.pntd.0005113.
33. Li J, Katiyar SK, Hamelin A, Visvesvara GS, Edlind TD. 1996. Tubulin genes from AIDS-associated microsporidia and implications for phylogeny and benzimidazole sensitivity. Mol Biochem Parasitol 78:289–295. https://doi.org/10.1016/s0166-6851(96)02628-x.
34. Thomas JH, Neff NF, Botstein D. 1985. Isolation and characterization of mutations in the beta-tubulin gene of *Saccharomyces cerevisiae*. Genetics 111:715–734.
35. Jung MK, Wilder IB, Oakley BR. 1992. Amino acid alterations in the benA (beta-tubulin) gene of *Aspergillus nidulans* that confer benomyl resistance. Cell Motil Cytoskeleton 22:170–174. https://doi.org/10.1002/cm.970220304.
36. Katiyar SK, Gordon VR, McLaughlin GL, Edlind TD. 1994. Antiprotozoal activities of benzimidazoles and correlations with beta-tubulin sequence. Antimicrob Agents Chemother 38:2086–2090. https://doi.org/10.1128/aac.38.9.2086.
37. Kwa MS, Veenstra JG, Roos MH. 1994. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. Mol Biochem Parasitol 63:299–303. https://doi.org/10.1016/0166-6851(94)90066-3.
38. Davidse LC, Flach W. 1977. Differential binding of methyl benzimidazol-2-yl carbamate to fungal tubulin as a mechanism of resistance to this antimitotic agent in mutant strains of *Aspergillus nidulans*. J Cell Biol 72:174–193. https://doi.org/10.1083/jcb.72.1.174.
39. Salát J, Braunfuchsová P, Kopecký J. 2001. Experimental infection of immunocompetent and immunodeficient mice with *Encephalitozoon cuniculi*. Folia Parasitol (Praha) 48:249–254. https://doi.org/10.14411/fp.2001.041.
40. Valenčáková A, Halanová M. 2012. Immune response to *Encephalitozoon* infection review. Comp Immunol Microbiol Infect Dis 35:1–7. https://doi.org/10.1016/j.cimid.2011.11.004.
41. GlaxoSmithKline. 2007. Albenza (albendazole) tablets prescribing information. GlaxoSmithKline, Research Triangle Park, NC.
42. Dayan AD. 2003. Albendazole, mebendazole and praziquantel. Review of non-clinical toxicity and pharmacokinetics. Acta Trop 86:141–159. https://doi.org/10.1016/s0001-706x(03)00031-7.
43. Sak B, Jandová A, Doležal K, Kváč M, Květoňová D, Hlásková L, Rost M, Olšanský M, Nurcahyo W, Foitová I. 2017. Effects of selected Indonesian plant extracts on *E. cuniculi* infection. Exp Parasitol 181:94–101. https://doi.org/10.1016/j.exppara.2017.07.014.
44. Didier ES, Stovall ME, Green LC, Brindley PJ, Sestak K, Didier PJ. 2004. Epidemiology of microsporidiosis: sources and modes of transmission. Vet Parasitol 126:145–166. https://doi.org/10.1016/j.vetpar.2004.09.006.
45. Seddon J, Bhagani S. 2011. Antimicrobial therapy for the treatment of opportunistic infections in HIV/AIDS patients: a critical appraisal. HIV AIDS (Auckl) 3:19–33. https://doi.org/10.2147/HIV.S9274.
46. Hofmannová L, Sak B, Jekl V, Mináriková A, Skorič M, Kváč M. 2014. Lethal *Encephalitozoon cuniculi* genotype III infection in Steppe lemmings (*Lagurus lagurus*). Vet Parasitol 205:357–360. https://doi.org/10.1016/j.vetpar.2014.07.008.
47. De Bosschere H, Wang Z, Orlandi PA. 2007. First diagnosis of *Encephalitozoon intestinalis* and *E. hellem* in a European brown hare (*Lepus europaeus*) with kidney lesions. Zoonoses Public Health 54:131–134. https://doi.org/10.1111/j.1863-2378.2007.01034.x.
48. Katzwinkel-Wladarsch S, Lieb M, Helse W, Löscher T, Rinder H. 1996. Direct amplification and species determination of microsporidian DNA from stool specimens. Trop Med Int Health 1:373–378. https://doi.org/10.1046/j.1365-3156.1996.d01-51.x.
49. Wolk DM, Schneider SK, Wengenack NL, Sloan LM, Rosenblatt JE. 2002. Real-time PCR method for detection of *Encephalitozoon intestinalis* from stool specimens. J Clin Microbiol 40:3922–3928. https://doi.org/10.1128/jcm.40.11.3922-3928.2002.
50. Dai J, Wang P, Adusumilli S, Booth CJ, Narasimhan S, Anguita J, Fikrig E. 2009. Antibodies against a tick protein, Salp15, protect mice from the Lyme disease agent. Cell Host Microbe 6:482–492. https://doi.org/10.1016/j.chom.2009.10.006.